PRINCIPLES IN SEROLOGIC GROUPING OF B. ABORTUS AND B. MELITENSIS. CORRELATION BETWEEN ABSORPTION AND AGGLUTINATION TESTS

STUDIES ON THE GENUS BRUCELLA NOV. GEN. II

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A series of absorption tests, carried out with B. abortus and B melitensis antiserums, resulted in a definite grouping of B. abortus and B. melitensis strains. A similar grouping ensued from a series of agglutination tests, in which eleven antiserums from rabbits, a monkey and a guinea-pig were used. The correlation, under diverse circumstances between the two sets of groupings, permits the inference that certain immutable laws govern serologic reactions, and although these laws remain elusive, we have been impressed by certain important principles involved in grouping.

Some preliminary tests with B. abortus and B. melitensis antiserums disclosed marked variations in the agglutinability of a number of authentic melitensis strains. In several melitensis antiserums, for example, the range of variability was so great that certain strains appeared to have little in common. Nevertheless, a relationship was apparent in that the strains of high titer in one antiserum perhaps group themselves as low titer strains in another antiserum. Thus while the variability was not identical, it was at least constant for the strains concerned. This suggested that definite principles might govern these variations. In the hope of elucidating these principles and of thus establishing the relationship among B. melitensis strains and their relationship to B. abortus strains, absorption tests were undertaken with abortus and melitensis antiserums. These experiments resulted in a four-fold grouping of the strains concerned.

PRELIMINARY DISCUSSION ON ANTIBODIES

The creation of antibodies is explained on the hypothesis that the bacterium consists of one or more components, each producing its specific agglutinins during the process of immunization. Biologically

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allied species may possess one or more components in common, hence the phenomenon of group or coagglutination. Castellani demonstrated that by saturating the antiserum with an emulsion of the coagglutinating organism, all these coagglutinins could be removed, leaving the specific agglutinins of the homologous strains intact. It was formerly believed that an antiserum could be exhausted of the specific agglutinins only by saturation with the homologous strain, but both Kruse and the co-workers, Andrewes and Inman, in their study of the serological races of the Flexner dysentery group, found that this was not the case. As the latter workers pointed out, "Of two strains A and B, A may be able completely to exhaust serum B, but B may be unable to do the same for serum A." Our own experience corroborates this point, and we have been able further to specify the condition under which occurs the phenomenon of a nonhomologous strain exhausting an antiserum, namely, when the two strains belong to the same serologic group, but are not identical, as will be demonstrated later.

In comparison to the stress laid on the structure of the bacterium in evoking the formation of antibodies, too little emphasis has been placed on the complexity of the serum itself. It is as if we mixed an acid in an alkaline solution and were to explain the process of neutralization by the composition of the acid regardless of the composition of the alkali. We find, for example, that the same bacterium may evoke the formation of quantities of antibodies in the serum of one species of animal, while it evokes but a feeble amount in a serum of another species;[1] yet we evade all responsibility of attempting to unravel the mystery from the point of view of the serum. We appease our curiosity with some selfevident platitude, such as the individuality of the animal, and thus veneer our ignorance with a gloss of logic. Why this individuality? Until serologic workers direct their investigations to unraveling the complexity of the serum, we cannot hope to fathom the marvelous mechanism of serologic reactions, nor can we offer any adequate explanation why a fourfold division of bacterial species results repeatedly from serologic classifications. Meningococcus (Gordon), pneumococcus (Cole and associates), tetanus (Tullock), Flexner dysentery (Andrewes and Inman),[2] typhoid (Weiss

[1] For example. one of our strains produced agglutinins to 1:20,000 in a monkey, and 1:2,000 in a rabbit.

[2] According to Andrewes and Inman, their "Y" or fifth race contains no specific antigenic component but presents a mixture of the component of the other four races. They say "we have failed fully to solve the antigenic structure of the 'Y' races. All that we have been able to do has been to obtain evidence of the presence in the race of most or all of the antigenic components which we have termed, V, W, X, and Z." (p. 36) Ref. 1.

and Hooker), streptococci (Dochez, Avery and Lancefield), influenza (Small-Dickson), and our own work on abortus-melitensis are included in this fourfold division. Is it not significant that the isolysins and iso-agglutinins of the human race also fall into four groups?

PRELIMINARY DISCUSSION ON ABSORPTIONS

In all our absorption tests we have proceeded on the principle that incomplete absorption of the nonspecific agglutinins, while it may yield interesting data, cannot establish definite laws. Unless the antiserum is absorbed to extinction of its nonspecific agglutinins, no subsequent reaction can be classed as distinctly specific. As a control, our absorbed serum was always tested with the absorbing strain along with our entire series of experimental organisms. If any agglutinins remained for the absorbing strain, the test was discarded. In practice we found it helpful to make a preliminary test with the absorbing strain, and if all of its agglutinins had not been removed the serum was reabsorbed. When the limit of extinction is reached, no further saturation with a nonspecific strain will effect a reduction in the titer of the serum for the specific agglutinins. On the other hand, as we have observed, an incomplete absorption, on becoming complete, may further reduce the specific titer 50%. It seems of vital importance, therefore, if we are to class residual agglutinins as specific that the nonspecific agglutinins should be absorbed to extinction.

Logically, the term specific is relative to the absorbing strain—for different bacteria may absorb different amounts and kinds, and in each case we term the residual agglutinins "specific." It may be possible that the definitely specific agglutinins of a bacterium can be measured only by successive saturations of its antiserum with different bacteria, each capable of removing quantitatively and qualitatively its own coagglutinins. But even then, there would be a minimum of residual agglutinins beyond which no bacterium except the homologous strain, or one of the same group, could exhaust the antiserum. We have not performed such tests and we use the term specific in its accepted sense of residual agglutinins after absorption with a nonhomologous strain.

TECHNIC

Antigens.—The strains were grown on peptic digest agar, the growth washed off with a few c c of formalinized salt solution and stored in the ice chest as stock emulsions. To 100 c c of formalinized salt solution the necessary amount of emulsion was added to match a standard in capacity containing about one

and one-half billion organisms. The same amount was added in each case whenever it became necessary to replenish the antigens. Although we use light suspensions throughout the experiments, we have since made use of opaque suspensions which have yielded excellent results. All agglutination readings were recorded after 18 hours in the incubator.

Method of Absorption.—The antiserum was diluted 1:10 with salt solution. An equal quantity of an emulsion of the absorbing antigen was added, making a dilution of 1:20. The tube was left 2 hours in the incubator and overnight in the ice chest, after which it was centrifuged one hour. The clear serum was decanted and a preliminary test made with the absorbing strain, to determine whether all of its agglutinins had been removed. If not, the serum was reabsorbed, using packed cells of the antigen in order not to alter the dilution of 1:20 The following day the clear serum was tested for agglutinins with 14 of our experimental strains, including the control. Thus we obtained evidence of relationship, not only between the absorbing and the homologous strains, but also for a number of other strains. Grouping at once became apparent.

Antiserums.—Although seven antiserums were employed in the course of our absorption investigations which covered over 400 tests, we submit the data from two of these, these two having been absorbed systematically by the greatest number of strains. Table 1 also contains the data of absorpt on tests from monkey antiserum. The magnitude of the task prevented us from pushing the absorption tests to completion in all the antiserums, but about 100 selected tests convinced us that the results paralleled each other. The antiserums of the classification submitted were made by immunizing rabbits, with both dead and living cultures, one with B. abortus 80, and the other with B. melitensis 7, these being our classical stock strains of B. abortus and B. melitensis.

RESULTS AND RECORDS OF ABSORPTION TESTS

It is quite evident that by absorbing an antiserum with one strain and then testing it with a number of other strains we may obtain one of three results. The coagglutinins may have been removed for all the remaining strains, thus giving a negative reaction throughout; they may have been removed for none of the remaining strains, thus giving a positive reaction throughout; or they may have been removed for some and not for others, thus giving a negative reaction for the former and a positive reaction for the latter. In the first and second cases we obtain no information as to grouping, all strains having followed an identical course. In the third case, however, a division into two groups is apparent. Let us assume, for example, that a certain serum absorbed by a certain strain was then positive for A, B and C, and negative for X, Y and Z, thus giving us two groups. Now another serum absorbed by the same strain, or if we choose the same serum absorbed by another strain, may leave A, B and Z negative, while X, Y and C are positive. We now have four groups. A and B have acted identically

throughout, and X and Y have clung together; C has fallen into a group by itself, and Z has done likewise. A third absorption with another serum or another strain may leave A and Y positive, and B, C, X and Z negative. We would now have as many groups as strains, for no two would have followed an identical course throughout.

Apparently this differentiation into groups is based on affinity for the same agglutinins. So long as two strains continue to follow parallel courses under various conditions, they are exhibiting like properties and may be assumed to possess similar components; for when the coagglutinins are absorbed from an antiserum for one of the strains, they are absorbed for the other; when the coagglutinins remain for the one, they likewise remain for the other. If this uniform behavior continues throughout a series of different types of tests, it is reasonably evident that the strains have a preponderance of something in common. Since they constantly react uniformly they naturally group themselves in the same categories.

At a special stage in our work, we became conscious of this group affiliation. Certain strains were exhibiting identical reactions (qualitatively) regardless of the antiserum or the absorbing strain used. There was, it is true, a quantitative difference—the titer when positive was higher or lower—but qualitatively they reacted in a uniform manner—their coagglutinins were either absorbed or were not absorbed under the same conditions. By checking the results, we found that the fourteen experimental strains fell into four groups with the greatest number in group 2.

These groups were as follows:

Group 1: Melitensis, 20; abortus, 80.

Group 2: Melitensis, 18, 19, 21, 2, 6, 8, 11, 655, 10.

Group 3: Melitensis, 7.

Group 4: Paramelitensis, 22; paramelitensis, 23.

Other strains tested irregularly distributed themselves in the various groups, the abortus strains invariably falling in group 1. Table 1 represents a portion of the data from which these groups were compiled. The results are expressed qualitatively (not quantitatively). The experimental strains are in the extreme left column and their reactions are to be read in a horizontal line. The strain and antiserum used in the absorption test are indicated at the top of the column and at the bottom, the groups to which they belong.

In endeavoring to analyze the principles involved in this grouping our data (as may be seen from the table) indicated that the strains

of one group could not exhaust the antiserum of another group; for example, no strain of groups 2, 3 or 4 could exhaust the antiserum of group 1. The antiserum of group 1 could be exhausted only by its homologous strain or by some other strain of group 1. This suggests that there is a specific component in each group which differentiates it from all other groups. On the other hand, it is apparently a common property for members within a group to exhaust the antiserum one

TABLE 1

QUALITATIVE RECORDS OF ABSORPTION TESTS REPRESENTING THE FOUR GROUPS OF B. ABORTUS AND B. MELITENSIS

Agglutinated with	Rabbit								
	Anti-melitensis 7 Absorbed with Melitensis 7	Anti-abortus 80 Absorbed with Melitensis 7	Anti-melitensis 7 Absorbed with Melitensis 11	Anti-abortus 80 Absorbed with Melitensis 11	Anti-melitensis 7 Absorbed with Melitensis 18	Anti-abortus 80 Absorbed with Melitensis 18	Anti-melitensis 7 Absorbed with Melitensis 19	Anti-abortus 80 Absorbed with Melitensis 19	Anti-melitensis 7 Absorbed with Melitensis 21
Melitensis 2	0	+	0	0	0	0	+	0	0
Melitensis 6	0	+	0	+	0	+	0	+	0
Melitensis 7	0	0	+	0	+	0	+	0	+
Melitensis 8	0	+	0	+	0	+	0	+	0
Melitensis 10	0	+	0	0	0	Not tested	0	0	0
Melitensis 11	0	+	0	0	0	+	0	0	0
Melitensis 18	0	+	0	0	0	0	0	0	0
Melitensis 19	0	+	0	0	0	+	0	0	0
Melitensis 20	0	+	0	+	0	+	0	+	0
Melitensis 21	0	+	0	+	0	+	0	0	0
Melitensis 22	0	0	+	0	+	0	+	0	±
Melitensis 23	0	0	+	0	+	0	+	0	±
Melitensis 655	0	+	0	0	0	+	0	0	0
Abortus 80	0	+	0	+	0	+	0	+	0
	Group 3 absorbed with group 3	Group 1 absorbed with group 3	Group 3 absorbed with group 2	Group 1 absorbed with group 2	Group 3 absorbed with group 2	Group 1 absorbed with group 2	Group 3 absorbed with group 2	Group 1 absorbed with group 2	Group 3 absorbed with group 2

+ signifies agglutination after absorption.
0 signifies no agglutination after absorption.
± signifies indistinct reaction.

for another without a reciprocal exhaustion taking place. Indeed in our limited investigations along this line, it was the prevailing case. We are not prepared to state that it is an obligatory relationship for one strain to be able to exhaust the antiserum of another strain in the same group, but we suspect that such may be the case. This implies a close relationship among the allied strains of the same group.

The second point revealed was that if a strain exhausted an antiserum of its coagglutinins for some strain in another group, it exhausted the coagglutinins for all strains in that group under the same absorption conditions; that is, the action was uniform (qualita-

tively) on the entire group. If the reaction were positive, the same principle applied. To illustrate the foregoing points, let us glance at the above grouping and suppose that strain 18 removed from antiserum 7 the coagglutinins for strain 20 (group 1), but not for strain 22 (group 4). Then strain 18 also removes the coagglutinins from antiserum 7 for strain 80 and for all other members of this group, but does not remove them for strain 23 or for any other members of this

TABLE 1—*Continued*

QUALITATIVE RECORDS OF ABSORPTION TESTS REPRESENTING THE FOUR GROUPS OF B. ABORTUS AND B. MELITENSIS

Antiserums						Monkey Antiserum					
Anti-abortus 80 Absorbed with Melitensis 21	Anti-melitensis 7 Absorbed with Paramelitensis 22	Anti-abortus 80 Absorbed with Paramelitensis 22	Anti-melitensis 7 Absorbed with Paramelitensis 23	Anti-abortus 80 Absorbed with Paramelitensis 23	Anti-melitensis 7 Absorbed with Melitensis 20	Anti-abortus 80 Absorbed with Melitensis 20	Anti-melitensis 7 Absorbed with Abortus 80	Anti-abortus 80 Absorbed with Abortus 80	Anti-melitensis 655 Absorbed with melitensis 7	Anti-melitensis 655 Absorbed with Abortus 80	Anti-melitensis 655 Absorbed with Melitensis 655
0	+	+	+	+	±	0	±	0	+	Not tested	0
+	Not tested	+	Not tested	+	±	0	±	0	+	+	0
0	+	?	+	0	+	0	+	0	0	0	0
+	+	+	+	+	±	0	±	0	+	+	0
+	Not tested	+	Not tested	+	±	0	±	0	+	Not tested	0
+	+	+	+	+	±	0	±	0	+	+	0
+	+	Not tested	Not tested	+	±	0	±	0	+	+	0
0	+	+	+	+	±	0	±	0	+	+	0
+	+	+	+	+	0	0	0	0	+	0	0
0	+	+	+	+	±	0	±	0	+	+	0
0	0	0	0	0	+	0	+	0	0	0	0
0	0	0	0	0	+	0	+	0	0	0	0
+	+	+	+	+	±	0	±	0	+	+	0
+	+	+	+	+	0	0	0	0	+	0	0
Group 1 absorbed with group 2	Group 3 absorbed with group 4	Group 1 absorbed with group 4	Group 3 absorbed with group 4	Group 1 absorbed with group 4	Group 3 absorbed with group 1.	Group 1 absorbed with group 1	Group 3 absorbed with group 1	Group 1 absorbed with group 1	Group 2 absorbed with group 3	Group 2 absorbed with group 1	Group 2 absorbed with group 2

group. We see, then, that a strain acts in a uniform manner on every member in another group, under the same absorption conditions.

We observed further (but we are not prepared to state this as a universal fact) that all strains in one group were likely to act in the same manner (qualitatively) on all members in another group when absorbed from the same antiserum. For example, continuing the above illustration, our data revealed that strains 19, 21 and 11 (same group as 18) also removed the coagglutinins from antiserum 7 for strains 20 and 80, but did not remove them for strains 22 and 23. The same principle asserted itself when five strains of group 2 were absorbed from group 1 antiserum. Here the reaction was positive for strains 20 and 80, and negative for strains 22 and 23 in all 5 cases.

We may state this tentatively as follows: All strains in one group tend to act in the same manner (qualitatively) on all strains in another group, when absorbed from the same antiserum. If this is true, there is a uniform action of group on group, which is more than our principle advocates, namely, the uniform action of each strain on the entire group.

Immediately a third principle manifested itself as an amendment to the preceding. Occasionally a group did not act in unison, but analysis revealed the definite condition under which this deviation occurred; namely, an absorbing strain might act in an irregular manner on members of its own group, thus bringing out their individual differences. For example, strain 18 when absorbed from antiserum 80 removed the coagglutinins for itself and for strain 2, but did not remove them for the remaining strains of the same group; that is, we have a mixture of positive and negative reactions for members of the same group when subjected to the same absorption conditions. It may be that the difference among members within a group is purely a quantitative one, that each possesses a preponderance of the specific agglutinins but varying amounts of the foreign coagglutinins, and this difference becomes manifest only when one of the group acts as the absorbing agent in removing the coagglutinins. It must be borne in mind that while a group absorbs irregularly for its own members the whole group is acted on uniformly by members of another group.

So far as we carried our experiments, we found no deviation from these three principles. We attempted to check our results by the following test: One of the workers planted from his own private stock 6 of the experimental strains and gave them to the other worker under fictitious lettering. In all 6 cases the strains were assigned to their proper groups and in 4 cases the exact organism was located. The latter point, however, is beyond the scope of our work. We cannot scientifically separate one strain from another in the same group, and the ability to do so is merely temporary and due to that intangible evidence which constant handling of a strain brings to a worker.

The three principles enunciated above may be briefly summarized:

1. An antiserum cannot be exhausted by strains of another group. It is always exhausted by its homologous strain and may be exhausted by other members of the same group.

2. A strain acts in a uniform manner (qualitatively) on all strains in another group under the same absorption conditions. This uniform action constitutes the basis for group affiliation.

3. Strains within the same group do not necessarily act in a uniform manner on one another under the same absorption conditions. This constitutes the basis for individual differentiation.

The degree of demarkation between any two groups is far from uniform. There is a wide difference between groups 1 and 3 and between groups 1 and 4, but the relationship between groups 1 and 2 is exceedingly close, and generally they follow identical courses only to be separated when some specific element comes into play. Frequently the strains of these groups interagglutinate to their full titers. Group 2 appears to be a transition between groups 1 and 3. It bridges their differences; it is related to both, but it is sharply separated from group 4. Group 3 may be taken as the type of the classic melitensis strain, from a serologic standpoint. Its agglutinins are in a large measure specific, and it is clearly defined from other types. Group 4 embraces strains of low agglutinability and of low antibody producing powers. They are fairly agglutinable in some antiserums if living suspensions are used, but all our work was carried out with formalinized suspensions. They appear to have few agglutinins in common with groups 1 and 2; they have a more pronounced absorbing effect in group 3 than their agglutinating titer might intimate. But agglutinating titer is not a criterion for absorbing titer. In comparing the percentage agglutinating with the percentage absorbing titer for the different groups, it was found that the percentage absorbing power of a group was frequently in excess of its percentage agglutinating power. The reverse was rare. On the whole, group 3 appears to be the pivot from which the strains radiate in one direction toward the abortus group, and in the other direction toward the highly specific paramelitensis group.

It has been stated that whenever B. abortus strains were tested they fell in group 1. We carried but one abortus strain, number 80, throughout all the tests. Into the same group fell melitensis 20. Under all absorption conditions these two strains followed identical courses except that there was a quantitative difference in titer. Melitensis 20 could exhaust the antiserum of B. abortus 80 and toward the end of our work when we prepared an antiserum for melitensis 20, we found that it could be depleted by B. abortus 80, thus giving a reciprocal exhaustion. We then reduced the absorbed dilution to 1 : 10, and 4 other B. abortus strains were now absorbed from melitensis 20 anti-

serum. All 4 exhausted it. One of these strains had been carried through about half the absorption test. We now found that melitensis 20 could exhaust its antiserum. Thus we had two B. abortus strains which could exhaust the antiserum of melitensis 20 and whose antiserum melitensis 20 could exhaust. Nevertheless, the 3 strains are not identical, so far as their histories go. Their titers, though approximate, are not identical in all antiserums. Their coagglutinins are not equally removed quantitatively by other strains. They grow differently both as regards speed and abundance. We are, therefore, forced to conclude that reciprocal exhaustion in dilutions as low as 1:10 is no criterion for identity of strains. This sounds illogical, but it does not exclude the possibility that specificity may be demonstrable in dilutions of 1:2 or 1:5. All that we conclude from these reciprocal absorption tests is the close relationship between B. abortus and one type of B. melitensis strains. What then is specificity? The fact that a nonhomologous strain can exhaust an antiserum casts a doubt on individual specificity as the exclusive possession of a single bacterium. It would appear that no bacterium is an isolated entity, all of its agglutinins provoked by immunization, are shared by some members in its group and out of its group. Each group may possess a separate primary attribute, but not each bacterium, and the sum total of these primary attributes constitutes the race; for example, the race of typhosus, of dysenteriae, of pneumococcus. The individuality of a bacterium would then appear to consist in its proportional share of the agglutinins of its race — the primary group agglutinins predominating — rather than in the possession of a specificity exclusively its own.

Andrewes and Inman,[3] in their masterly article on the Flexner dysentery types, used a quantitative method in their absorption tests. They determined the number of bacteria in their absorbing emulsions and diluted these for two absorbing doses, one containing approximately 1,000 million organism, and the other from 20,000 to 30,000 million organisms. They advocate a quantitative method. We have not been convinced of the advantage in determining the number of organisms in the absorbing dose—except its interest from the experimental point of view. It would be vexatious in routine work even if emulsions were kept in stock, and moreover its result might be fallacious. If our principle is correct that the absorbing strain must

[3] Medical Research Committee Special Report No. 42, 1919.

remove all the coagglutinins it is capable of absorbing — that is, to extinction of itself—there could be no fixed doses for any one strain, because its absorbing capacity varies with the potency of the serum and its relationship thereto.

When dense doses are required there seems to be less hindrance to the progress of the reaction, if absorbed fractionally. This, however, is not obligatory.

GENERIC CLASSIFICATION

The American Committee on the Classification of Bacterial Types [4] decided that "B. abortus may for the present be left in this genus (Bacterium) in spite of its peculiar oxygen relations." The genus Bacterium of the Bacteriaceae family constitutes the colon-typhoid-dysentery group. It would seem that this genus is already encumbered with sufficiently diversified types without the addition of B. abortus.

If in reality a classification is a scheme destined to convey some adequate idea of mutual relationships, should not its genera be so apportioned that each genus may be narrowed to a type, embracing individuals with fairly limited common characteristics and common differentiations from other types; thus, one genus should not include organisms with such widely varied specificity as B. coli, B. typhosus, B. dysenteriae and B. abortus, although all these would still be united in a common family. If, as is the case in the above genus, the "species" is left as the sole vehicle for differentiation (for the term subgenus is a useless encumbrance), a classification becomes an empty nomenclature, a mere vocabulary with which the sophisticated student may terrify the uninitiated scholar.

We advocate, therefore, that B. abortus be removed from the genus Bacterium, which includes the colon, typhoid and dysentery organisms, and we suggest that the abortus melitensis group be given separate rank as the genus "Brucella" (from Bruce who isolated the original melitensis organism, later identified by Nègre and Raynaud, as Micrococcus paramelitensis.[5]) The 4 groups as above formulated would then each embrace a number of allied species, and if it became expedient to establish subgroups, they would probably range themselves as varieties of some species.

[4] Jour. Bacteriol., 1917, 2, p. 546.
[5] Compt. rend. Soc. de biol., 1912, 72, p. 791 and 1052.

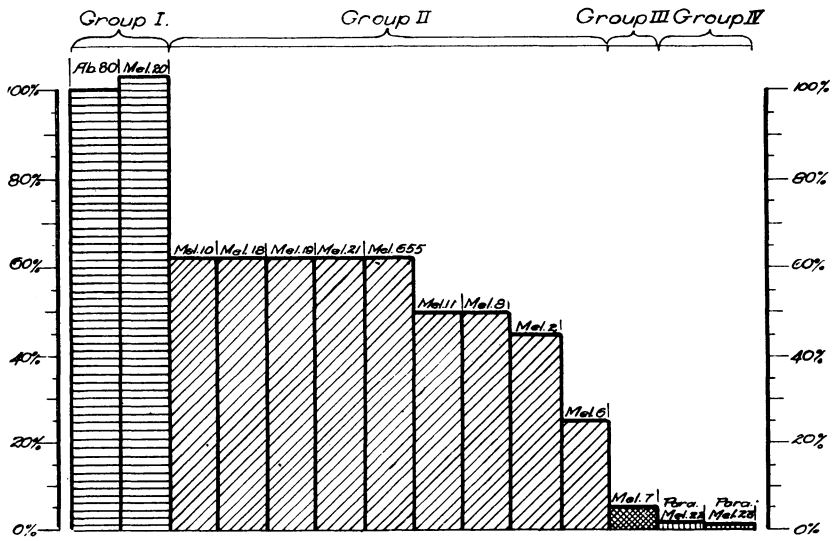
RESULTS AND RECORDS OF AGGLUTINATION TESTS

The experimental strains were agglutinated in 1 monkey, 1 guinea-pig and 9 rabbit antiserums. All 4 groups were represented by these 11 antiserums. We also tested the strains in the serums of 2 cows and 3 hogs suffering from natural abortion disease. Separate references will be made to these tests.

In addition to the experimental strains used in the absorption tests, we carried 15 other B. melitensis strains through all the tests and 35 B. abortus strains were agglutinated in 6 of the antiserums.

B. abortus and B. melitensis unlike B. typhosus, for example, are not strong antibody producers. Our B. abortus antiserums ranged from 1:2,000 to 1:4,000 and our B. melitensis antiserums approximated 1:2,000 except that

Chart 1.—Agglutinogenesis of group 1 antiserum. Columns of like marking represent different strains of the same group.



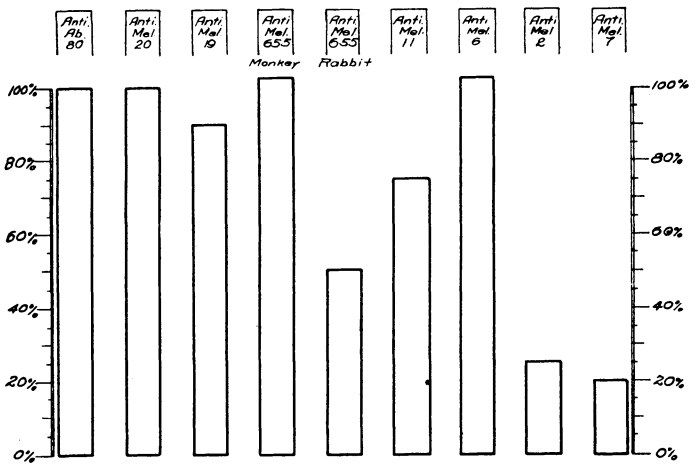
Abortus 80 antiserum (group 1). Percentage agglutination for 14 strains. The titer for the homologous strain is 100 per cent.

of the monkey which was active in a dilution of 1:20,000 and the so-called paramelitensis of group 4 which did not yield an antiserum beyond 1:200 in either a rabbit or a guinea-pig.

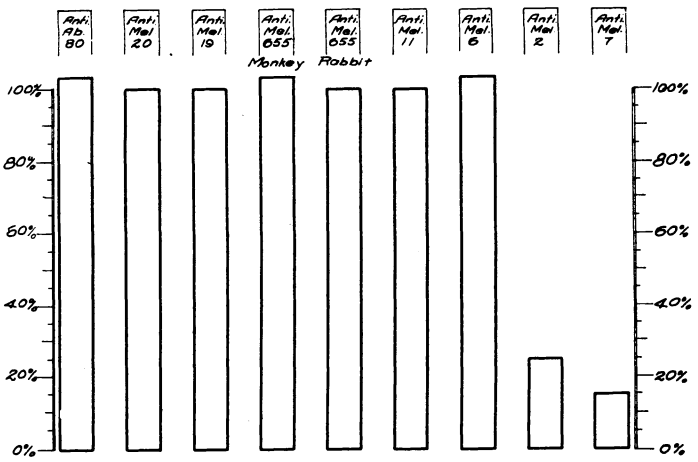
In expressing our results graphically, we have adopted the column and percentage method used by Andrewes and Inman.[3] As these workers pointed out, actual figures are not comparable owing to the different titers of various antiserums, whereas results are readily comparable if expressed as percentages of the titers for the homologous strains. The titer for the homologous organism is taken as 100% and the proportional titers for others are expressed as percentages of this. Thus, if a strain reacts to 1,000 in an antiserum which flocculates the homologous organism to 2,000, the former's titer is expressed as 50%. Occasionally a strain reacts beyond the titer of the homologous organism, in which case the percentage is expressed above the 100 mark.

In presenting the results which follow we shall discuss first, the action of the antiserums of each group on the various strains, and then the agglutination of the strains of each group in the various antiserums.

Chart 2.—Agglutination of group 1 strains in 9 antiserums. Compare columns above with columns below for action of same antiserum on two strains of group 1.



Percentage agglutination of abortus 80 bacterium (group 1) in 9 antiserums. The titer of each antiserum for its homologous strain is 100 per cent.

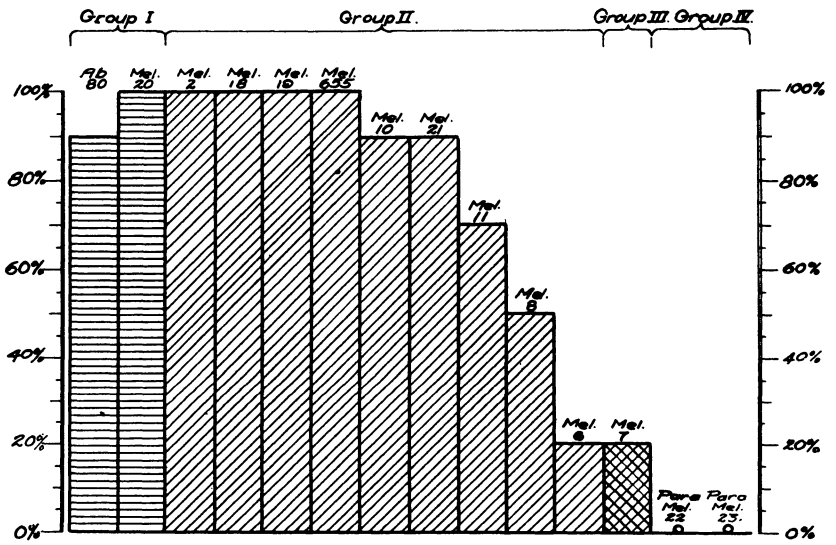


Percentage agglutination of Melitensis 20 bacterium (group 1) in 9 antiserums. The titer of each antiserum for its homologous strain is 100 per cent.

Group 1 Antiserums.—A potent antiserum of this group was characterized by definite gradations in the titer limits for the strains of the four groups. Group 1 strains agglutinated 100%. Group 2 averaged 60%, but in some of

the less potent antiserums, such as those not exceeding 1:2,000, these strains agglutinated to the full titers, thus making no distinction in agglutinability for groups 1 and 2, a situation which will repeat itself later on. Group 3 strains did not react beyond 5%, and group 4 strains 1% or less. In those antiserums in which group 2 strains reacted to the full titer, there was no proportional change in the titers for groups 3 and 4. Chart 1 represents a potent antiserum of group 1 showing the percentage agglutination for the experimental strains. In this antiserum the agglutination of group 2 averages 60%. Chart 9 represents the more common antiserum of group 1 in which the group 2 strains are flocculated to the titer limits. Strains 2 and 6 are somewhat irregular in most antiserums, but the absorption tests assign them to group 2.

Chart 3.—Agglutinogenesis of group 2 antiserum (rabbit). Columns of like markings represent different strains of the same group.



Melitensis 19 antiserum (group 2). Percentage of agglutination for 14 strains. The titer for the homologous strain is 100 per cent.

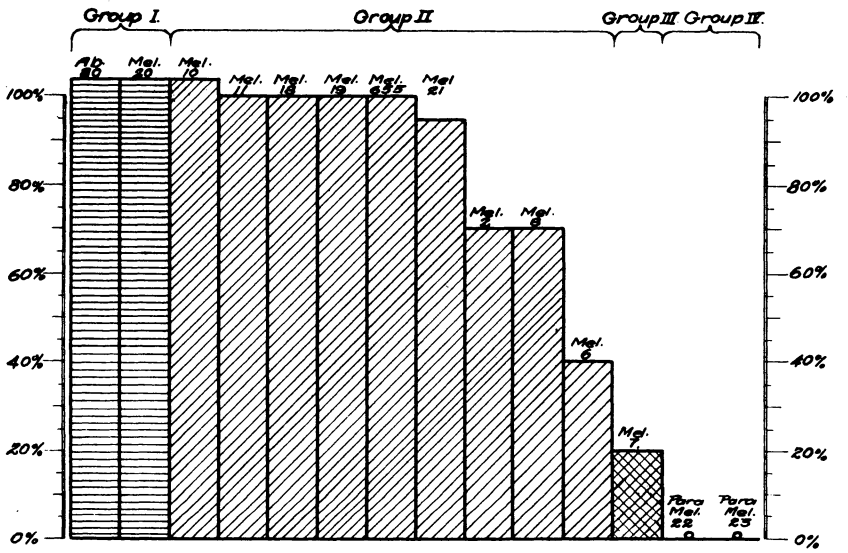
The 35 abortus strains showed a uniform range of 100% or thereabouts in all antiserums of group 1 and apparently represent a uniform group. Chart 10 shows these B. abortus strains in a group 1 antiserum.

Group 1 Strains.—Whatever may be the inhibitory forces which prevent a group 1 antiserum from flocculating the strains of groups 3 and 4 to any marked degree, the same forces are in evidence when the strains of group 1 are agglutinated in the antiserums of groups 3 and 4. About 40 B. abortus strains and B. melitensis 20 of group 1 could not react beyond 20% in group 3 antiserum. As group 4 (so-called paramelitensis) did not yield an antiserum in excess of 1:200, we limited our tests in this antiserum. Its agglutinins for group 1 fell below its titer. Group 2 antiserums generally agglutinated group 1 strains to close to the titer limits. It will be seen that B. melitensis 2 anti-

serum was not very potent for group 1 strains, showing a similar irregularity to that of group 1 antiserum for melitensis 2 strain. Chart 2 shows the agglutination of 2 unselected strains of group 1 in 9 antiserums.

Group 2 Antiserums.—These antiserums generally show no fundamental difference in the titers for strains of groups 1 and 2. They do not necessarily flocculate all strains to the titer limits, but the difference in degree of agglutinability is not sufficiently pronounced to establish a basis for separating the groups. Miss Evans'[6] y f strain—our B. melitensis 11—was of this group and from the reaction of its antiserum she concluded that "the agglutination reactions in Bacterium melitensis antiserum can distinguish Bacterium abortus from

Chart 4.—Agglutinogenesis of group 2 antiserum (monkey). Columns of like markings represent different strains of the same group.



Melitensis 655 antiserum (monkey), group 2. Percentage of agglutination for 14 strains. The titer for the homologous strain is 100 per cent.

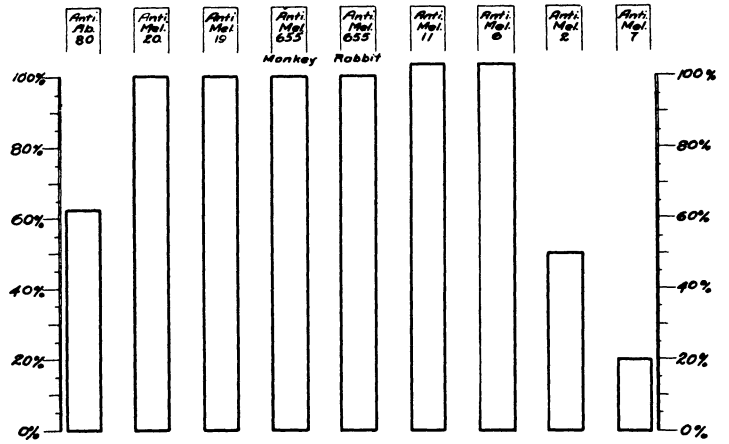
Bacterium melitensis only when the agglutinating strength of the serum for both species is known." Other workers (Kennedy[7]) who have found a close relationship in the reactions for both species, were probably working with group 1 strains.

It can be stated that whenever there is reciprocal agglutination approximating the titer limits in abortus and melitensis antiserums, the homologous strains must belong either to group 1 or to group 2. These groups cannot be separated from each other by absorption with a group 3 or a group 4 strain. They follow similar courses in such cases. They can be differentiated by reciprocal absorptions with their own group strains and antiserums. The distinction is gen-

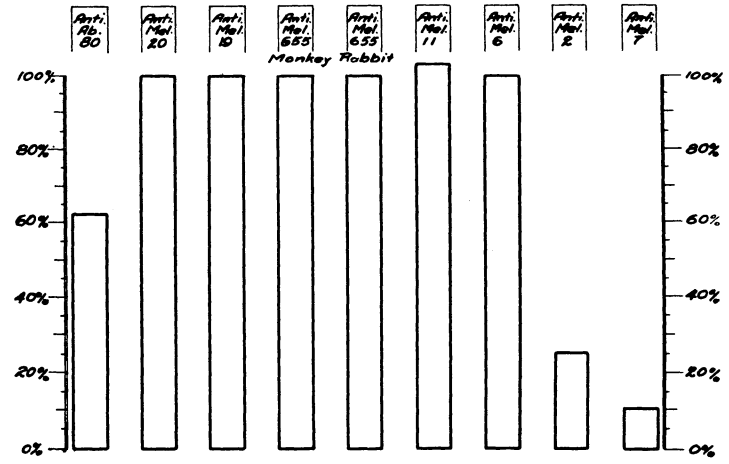
[6] Jour. Infect. Dis., 1918, 22, p. 580.
[7] Jour. Roy. Army Med. Corps, 1914, 22, p. 9.

erally very delicate. All strains in this group are not equally agglutinable. Strains 2, 6 and 8 may fall considerably below the titer limits. Indeed the repeated irregularities of strains 2, 6 and possibly 8 (for which we had no

Chart 5.—Agglutination of group 2 strains in 9 antiserums. Compare columns above with columns below for action of same antiserums on two strains of group 2.



Percentage of agglutination of melitensis 18 bacterium (group 2) in 9 antiserums. The titer of each antiserum for its homologous strain is 100 per cent.



Percentage of agglutination of melitensis 655 bacterium (group 2) in 9 antiserums. The titer of each antiserum for its homologous strain is 100 per cent.

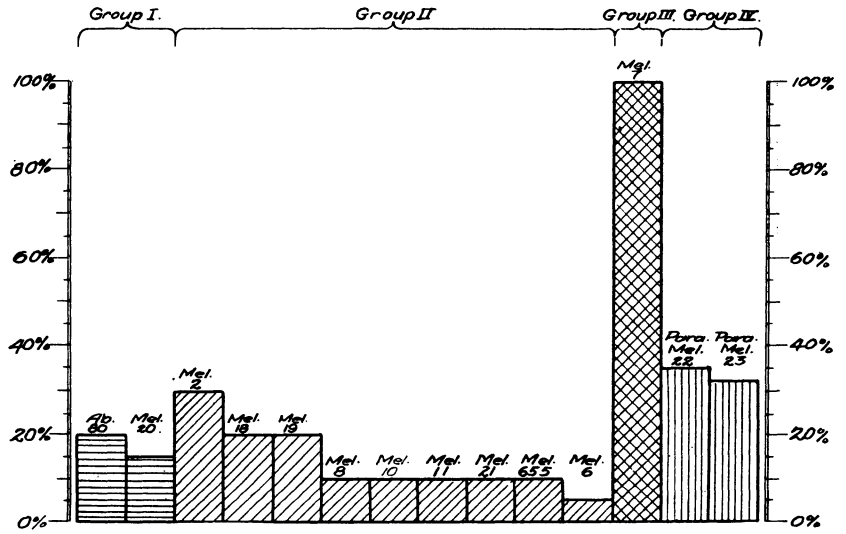
antiserum) suggest that their antigenic structure, if investigated, might justify placing them in a subdivision of group 2. Separately treated they would not distort the uniformity of the group nor distract the mind by constant reference to their irregularities. The titer of group 2 antiserum for group 3 strain

averages 20% except in antiserum 6 where it reacts to the titer limit. There are no demonstrable agglutinins in group 2 antiserums for group 4 strains except in antiserums 2 and 6 where there is a slight reaction.

Charts 3 and 4 show the titers of two antiserums of this group for the experimental strains. Chart 3 is a rabbit antiserum with a titer of 1:2,000 and chart 4 a monkey antiserum with a titer of 1:20,000. It will be seen that the gradations are fairly uniform in the two antiserums in spite of the striking differences in their titers and the fact that the immunization was made with 2 different strains of group 2.

Group 2 Strains.—As stated above, the strains of group 2 are agglutinated to about 60% or may be flocculated to 100% in group 1 antiserum. In group 3 antiserum they agglutinate from 10% to 20% of the full titer. Thus again we

Chart 6.—Agglutinogenesis of group 3 antiserum. Columns of like marking represent different strains of the same group.



Melitensis 7 antiserum (group 3). Percentage of agglutination for 14 strains. The titer for the homologous strain is 100 per cent.

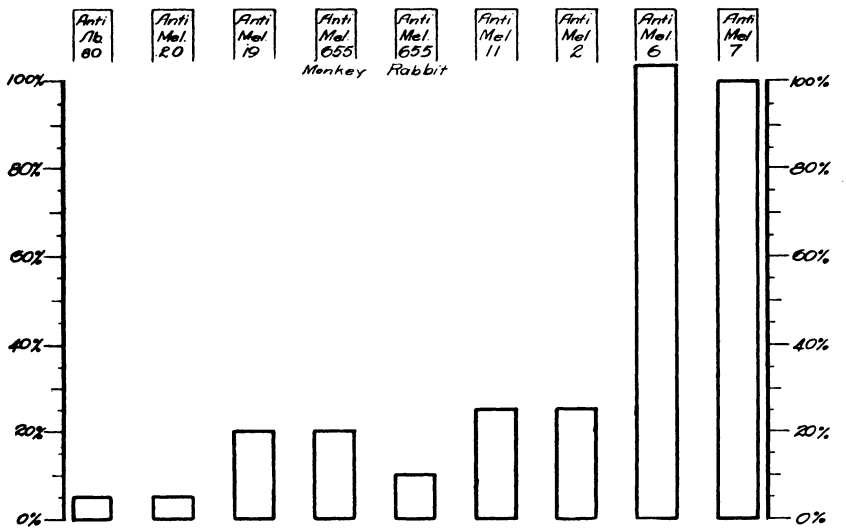
see the same inhibitory forces at work, confining the reaction of a group 2 antiserum on a group 3 strain and conversely the reaction of a group 3 antiserum on a group 2 strain to about 1/5 of their respective titers. In the weak antiserum of group 4 all strains of group 2 reacted to 1:200 which was the titer limit. Chart 5 shows the percentage agglutination of 2 strains of group 2 in 9 antiserums.

Group 3 Antiserum.—The foregoing summaries have necessarily overlapped the reactions for groups 3 and 4. The agglutinins for group 3, which measured 100% for itself, are low for strains of groups 1 and 2, averaging about 1/5 of the titer limit. Melitensis 2 runs somewhat higher and melitensis 6 slightly lower than the other strains. Groups 1 and 2 cannot be separated from each other by agglutination in group 3 antiserum though they can be differentiated instantly from group 3 itself. On the other hand, group 3 is the only antiserum in which there is a fair agglutination for strains of group 4. These

readily react to at least 30% of the titer. As living suspensions they may agglutinate to 100% in this antiserum. Chart 6 shows the percentage agglutination of group 3 antiserum for the experimental strains, and chart 9 for the series of B. abortus strains which reacted from 10% to 20%.

Group 3 Strain.—This strain (our 7) probably the M. pseudomelitensis of Sergent, Gillot and Lemaire,[8] reacts to 5% in group 1 and to about 1/5 of the titer in group 2 antiserums, and hence it is readily separated from them both. Melitensis 6 antiserum is a striking exception. It agglutinates group 3 to its titer limit. This is especially irregular because the antiserum of group 3 shows its minimum reaction on strain 6. Group 3 strain reacted to the full titer of 1:200 in the group 4 antiserum. Chart 7 shows the percentage agglutination of a group 3 strain in 9 antiserums.

Chart 7.—Agglutination of group 3 bacterium in 9 antiserums.



Percentage of agglutination of melitensis 7 bacterium (group 3) in 9 antiserums. The titer of each antiserum for its homologous strain is 100 per cent.

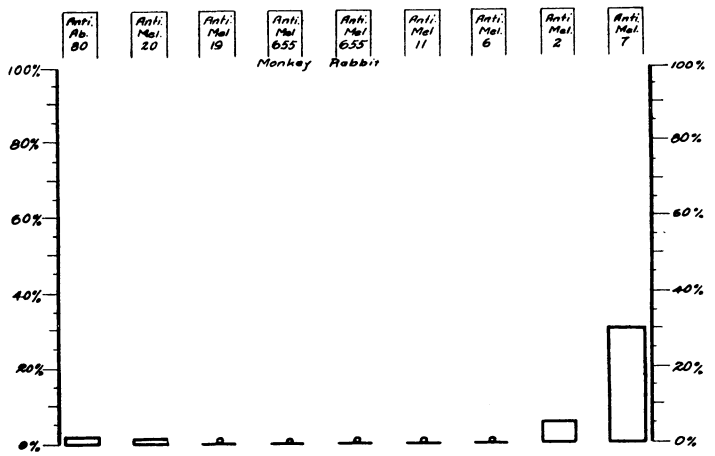
Group 4 Antiserum and Strains.—This low titer antiserum did not show any differential reaction for the various groups except that group 1 ran slightly below the titer. The strains of this group are inagglutinable in most antiserums. They show a slight reaction in group 1 antiserums and in melitensis 2 and melitensis 6 antiserums of group 2 in addition to their reaction in group 3 antiserum.

It will be seen from the subject matter presented above that we obtain striking gradations in agglutinability whether we consider the reaction of the antiserum on strains of the different groups or the reaction of strains in the antiserums of different groups. Moreover, these gradations coincide with the groups established by the absorption tests. We may briefly summarize these gradations:

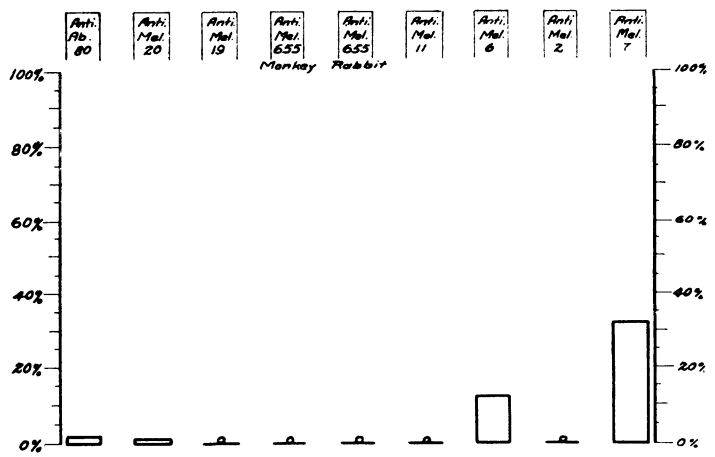
[8] Ann. de l'Instit. Pasteur, 1908, 22, p. 209.

Group 1 antiserum does not generally differentiate between the strains of groups 1 and 2, though in an occasional potent antiserum it may do so. It agglutinates group 3 weakly and is agglutinated weakly by group 3. It shows a minimum reaction for group 4.

Chart 8.—Agglutination of group 4 strains in 9 antiserums. Compare columns above with columns below for action of same antiserums on two strains of group 4.



Percentage of agglutination of paramelitensis 22 bacterium (group 4) in 9 antiserums. The titer of each antiserum for its homologous strain is 100 per cent.



Percentage of agglutination of paramelitensis 23 bacterium (group 4) in 9 antiserums. The titer of each antiserum for its homologous strain is 100 per cent.

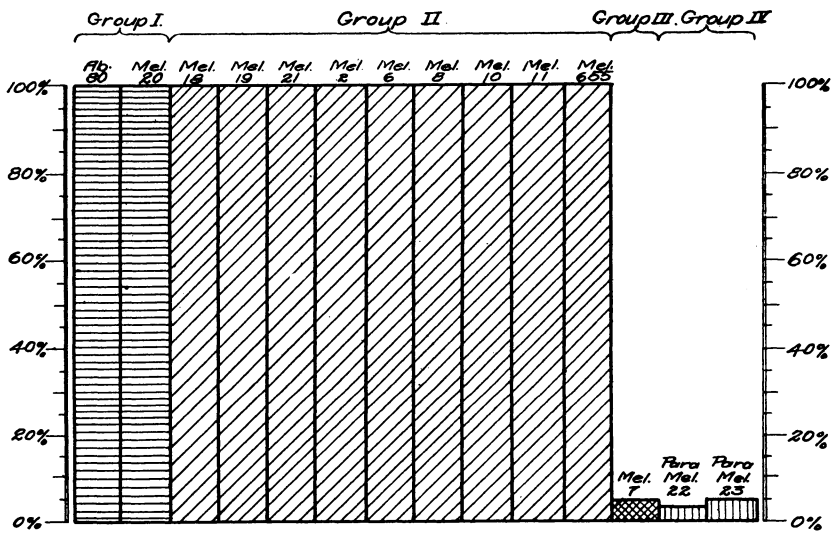
Group 2 antiserum shows no vitally specific differentiation between the strains of groups 1 and 2. It acts weakly on group 3 (except in antiserum 6) and is weakly acted on by group 3. It has no agglutinins for group 4 except in antiserums 2 and 6.

Group 3 sharply differentiates itself from groups 1 and 2, and is equally sharply differentiated by them (except in antiserum 6). It agglutinates group 4 to at least 30% of its titer.

Group 4 is differentiated by its prevailing inagglutinability in most antiserums and its inability to produce a potent antiserum in either rabbits or guinea-pigs.

The studies of Sergent and his co-workers,[8] who reported finding "para" and "pseudo" melitensis strains, probably foreshadowed our grouping. Nègre and Raynaud[5] identified one of Bruce's original organisms and gave it the name of paramelitensis. They also reported a race intermediate in agglutination between the "para" and the true melitensis.

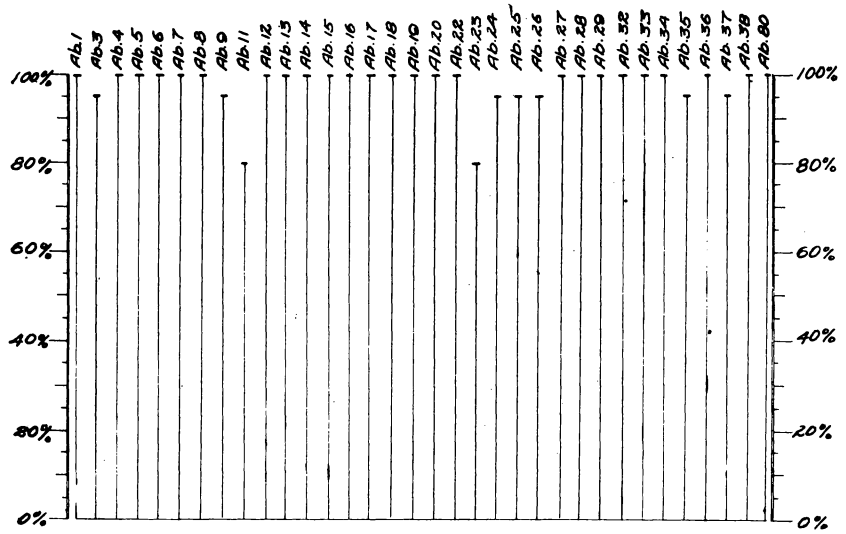
Chart 9.—Agglutinogenesis of group 1 antiserum. Columns of like marking represent different strains of the same group.



B. melitensis 20 antiserum (group 1). Percentage of agglutination for 14 strains. The titer for the homologous strain is 100 per cent.

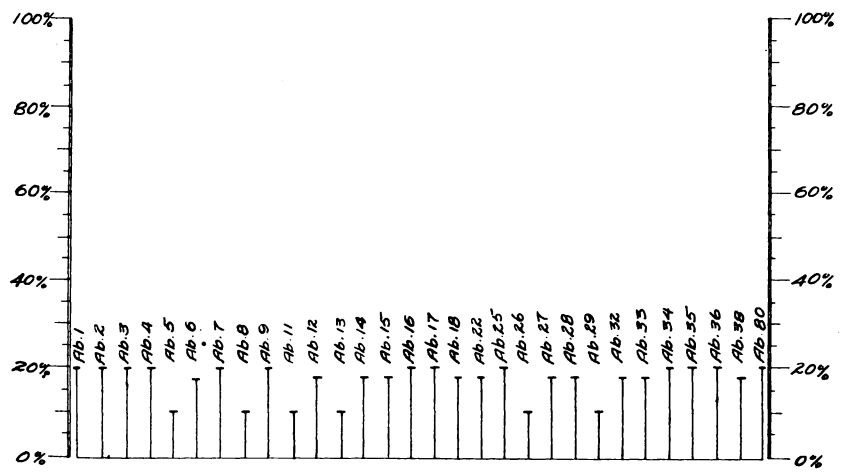
Through the courtesy of Dr. J. Traum, University of California, we obtained antiserums from 2 cows and 3 hogs suffering from natural abortion disease. All these serums, except that of one cow, reacted to 1:200 with B. abortus. The excepted cow showed no reaction to B. abortus nor to B. melitensis strains. There was, however, in unheated serum a faint sedimentation with both group 4, the so-called paramelitensis strains. The other cow, on the contrary, yielded an antiserum which flocculated all strains of groups 1 and 2 to the titer limit, but showed no reaction for strains of groups 3 and 4. The 3 hog antiserums flocculated B. abortus and showed a weak reaction for one or more B. melitensis strains, that is, 2 of the hogs gave a reaction for but one (not the same) B. melitensis strain, and the third for 6 melitensis strains. Group 3 was not agglutinated by any of the 5 antiserums. It would seem that the animals, except one cow, were infected by a group 1 strain, the abortus group. From

Chart 10.—Agglutination of B. abortus strains in group 1 antiserum.



B. abortus 14 antiserum (group 1). Percentage of agglutination for B. abortus strains. The titer for the homologous strain is 100 per cent.

Chart 11.—Agglutination of B. abortus strains in group 3 antiserum.



B. melitensis 7 antiserum (group 3). Percentage of agglutination for B. abortus strain. The titer for the homologous strain is 100 per cent..

the above data we see that in immune serums naturally or artificially produced we obtain a reaction for both B. abortus and B. melitensis strains, which corroborates fully the observations of Evans[6] and Kennedy[7] with bovine serum and milk whey.

SUMMARY

Unless an antiserum is absorbed to extinction of the absorbing strain, the residual agglutinins cannot be classed as specific.

A series of absorption tests with formalinized suspensions in B. abortus and B. melitensis antiserums led to a fourfold grouping of 14 B. abortus and B. melitensis strains. Groups 1 and 4 were represented by 2 and group 3 by 1 strain, the majority fell in group 2. All B. abortus strains belonged serologically to group 1. Groups 1 and 2 are closely related. They are sharply defined from groups 3 and 4.

The grouping revealed these principles:

1. An antiserum cannot be exhausted by strains of another group. It is always exhausted by its homologous strain, and may be exhausted by other members of the same group.

2. A strain acts in a uniform manner (qualitatively) on all strains in another group under the same absorption conditions. This uniform action constitutes the basis for group affiliation.

3. Strains within the same group do not necessarily act in a uniform manner on one another when absorbed from the same antiserum. This constitutes the basis for individual differentiation.

In conforming to the main classification adopted by the Society of American Bacteriologists, we suggest that B. abortus and B. melitensis group be given generic rank in the Bacteriaceae family as the genus "Brucella."

A series of agglutination tests in B. abortus and B. melitensis antiserums disclosed gradation in titer limits for the different strains and the gradations were constant for the same strains in the various antiserums. It was found that the sets so formed correlated with the groups resulting from the absorption tests.

The serums of cows and hogs suffering from natural abortion disease may also react to both B. abortus and B. melitensis organisms.